

Exhibit # 525

EFFECTS OF METHANOL ON THE FERTILIZATION
PROCESS OF CHUM SALMON (*ONCORHYNCHUS KETA*)

by

P.C. Craig

Aquatic Environments Limited

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ABSTRACT

The toxicity of methanol to the fertilization process of chum salmon was investigated because methanol from pipeline-related activities is a potential contaminant in northern watercourses. Both gametes and fertilized eggs were subjected to methanol concentrations (0.001 to 10%) for brief periods. An exposure time of 32 sec allowed an examination of effects on gametes; a 30 min exposure included possible lethal or sublethal effects of methanol uptake during imbibition (water hardening).

Results demonstrated that eggs, sperm and early embryos can withstand brief exposure to methanol concentrations up to and including 1%. These levels did not significantly effect 1) fertilization success, 2) survival to hatching, 3) hatching time, 4) alevin size at hatching, or 5) physical deformities among alevins. However, a 10% methanol concentration was lethal in most cases.

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INTRODUCTION

Methanol (methyl alcohol) is one of the potential contaminants which may leak or spill into northern watercourses from pipeline construction and testing. Large quantities of this chemical will serve as an antifreeze in the hydrostatic testing of the gas pipeline proposed by Canadian Arctic Gas Study Limited. The need for a quantitative assessment of methanol toxicity to fishes is apparent. An initial investigation of this problem was carried out by McMahon and Cartier (1974). The present study was conducted in response to recommendations by the U.S. Department of the Interior in their Draft Environmental Impact Statement (1975).

The purpose of this study is to examine the toxic effects of methanol on the eggs and sperm of a salmonid fish during fertilization. In the event of a methanol spill, the life cycle of these fish is presumably at its most vulnerable stage at the time of spawning. It has been speculated that sperm and ova might be extremely sensitive to small concentrations of methanol in the environment (McMahon and Cartier, 1974).

The salmonid used in this study was the chum salmon (*Oncorhynchus keta*) collected in British Columbia. Although not an abundant species in Arctic waters, chum have been taken in the Colville, Sagavanirktok and Mackenzie rivers (McPhail and Lindsey, 1970; Yoshihara, 1973). There is a large run of chum salmon in the Procupine River drainage (Elson, 1975).

EXPERIMENTAL DESIGN

During salmonid spawning, the time between shedding of gametes and fertilization of ova is extremely brief. The copious quantity of sperm and eddies within the nest ensure that most eggs are fertilized within 15-30 sec (Withler, personal communication). During this time and for the next half hour or so, the eggs imbibe water and become "water hardened" (Figure 1). Morley and Withler (1969) estimate that eggs increase in volume by 14% during water hardening.

With respect to the potential effects of methanol on the fertilization process, there are two important periods. First, the brief period prior to penetration when both sperm and unfertilized ova are exposed to methanol and second, the period after penetration when the eggs imbibe water and, presumably methanol. There is some information available concerning the movements of materials through egg membranes during the latter period. For example, the transfer of high concentrations of cadmium through herring egg capsules is extremely rapid during imbibition, and furthermore, some of this contaminant passes through the vitelline membrane and enters the yolk (Rosenthal and Sperling, 1974; Rosenthal and Alderdice, in preparation). These authors also found that these eggs became impermeable to cadmium at later stages, so that most of the cadmium which entered the egg did so during water hardening. Hence, an exposure time of 32 sec was chosen to examine the effects of methanol on gametes alone, and a longer

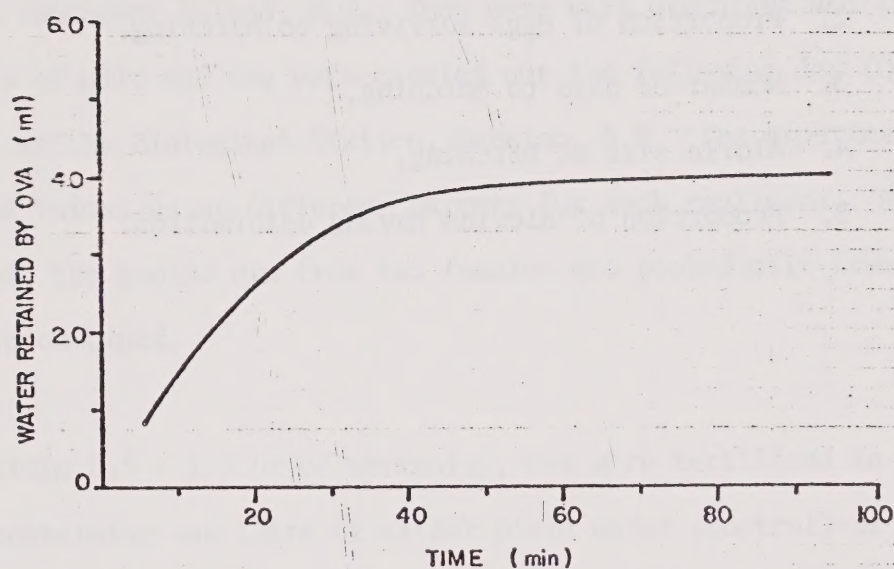


FIGURE 1. Water uptake by sockeye salmon ova. Graph represents water retained in 25 ml portions of newly-spawned ova after immersion in water. (Redrawn from Morley and Withler, 1969).

exposure of 30 min was chosen to show the effects of methanol uptake during water hardening.

After methanol treatment, the eggs were incubated and observed at intervals through hatching. The following observations were made:

1. Proportion of eggs fertilized,
2. Proportion of eggs surviving to hatching,
3. Number of days to hatching,
4. Alevin size at hatching,
5. Proportion of alevins having deformities.

MATERIALS AND METHODS

Eight chum salmon (four males and four females) were collected alive by seine and dipnet from spawning grounds in Nanoose and Bonell creeks on Vancouver Island, B.C. They were held overnight and the treatments of milt and ova were carried out the following day (October 29, 1975) at the Biological Station, Nanaimo, B.C. The experiment was replicated twice, using different parents for each replicate. Within replicates, the pooled ova from two females and pooled milt from two males were combined.

Within 0.5 - 1.5 hr of stripping, ova were fertilized in plastic buckets containing one litre of either plain water (control) or one of five methanol solutions (treatments): 0.001%, 0.1%, 0.1%, 1.0% and 10.0% methanol volume. The methanol solutions were drawn in a randomized sequence. The first and last tests in each series were controls so that a systematic variable, such as decreasing fertilizability of ova over time, would be identifiable. As in previous tests (McMahon and Cartier, 1974), a single batch of reagent grade methanol of known analysis was used for all tests (Appendix Table 1). All test dilutions were stored in a 9.5°C water bath for a least 1.5 hr prior to testing to dissipate heat of reaction.

The experiment was further sub-divided by subjecting the gametes and fertilized eggs to the methanol solutions for two time periods: 32 sec and 30 min. The procedure was to add simultaneously 2 ml of milt and approximately 50 ml of ova to the control or methanol solution and to stir briefly. Rinsing of the eggs was initiated after 15 sec in the first series and after 30 min in the second series. The time involved in thorough rinsing and subsequent flooding with fresh water was 17 sec (in each of three timings). While these seconds involved in rinsing are a negligible addition to the 30 min exposures, they double the exposure time in the short-exposure series. The latter is therefore referred to as a 32 sec exposure time.

After rinsing, the eggs from each test were placed in randomized compartments of two incubator trays (Heath vertical stack incubator). The eggs were incubated at 9.3°C (0.33°C , standard deviation) for the duration of the experiment. Temperatures were recorded with a Taylor 7-day continuous recorder. Daily temperature was calculated by averaging the temperature recorded at four times during each day (0600, 1200, 1800, 2400 hr). Time-temperature units are expressed as "C-degree days" of incubation.

At the 300 C°-Day stage, the 'eyed' eggs were shocked with a fine spray of water to reveal which eggs were infertile (the yolk material of infertile eggs coagulates and turns white). These and all other dead eggs were examined for the presence or absence of embryos after being cleared with Stockards' Solution. Once hatching began, alevins

were counted daily and removed from the trays. Every third or fourth alevin (for a total of $n=30$) was measured to the nearest 0.5 mm. All alevins were preserved in 10% formalin for later examination for physical deformities.

RESULTS

Fertilization Success

Methanol concentrations up to and including 1% did not significantly affect fertilization success. When the exposure period was 32 sec, 96% or more of the ova in combined replicates were fertilized (Table 1, Figure 2). Slightly fewer ova were fertilized when the exposure period was extended to 30 min (including control tests), but even then the fertilization rate was at least 90% (Table 2 , Figure 2). Within the 0.001 - 1.0% range of methanol concentrations, the highest concentration (*i.e.* "worst case" treatment) was compared statistically to the control data. In both exposure series, there was no significant difference between the results obtained from controls and the 1% methanol treatments.

On the other hand, a methanol concentration of 10% was lethal. Fertilization was impaired for nearly all ova subjected to this concentration for 30 min. Embryos were present in only 4 (1.5%) of the 267 eggs tested. Even when the gametes were exposed to a 10% methanol concentration for only 32 sec, mortality was high. Only 28% (range 10 - 49%) were fertilized.

It is possible that somewhat more eggs were fertilized than shown above. The criterion for successful fertilization was the presence of an embryo within the egg, but embryos are not large enough to be detected by eye during the first 10 days after fertilization. Thus some embryos

TABLE 1. Results of methanol treatments for the 32 sec exposure series. Length measurements are based on a sample size of 30 (except in one case, Replicate 2 - 10%, where n=12).

Repli- cate	% Methanol	ALEVINS						
		OVA		% Survival to hatch	Mean hatching (C°Days)	Length (mm)		S.D.
		N	% Fertil- ized			mean	range	
1	Control ¹	99	96	96	539.8	24.3	(23.0-25.0)	0.4
	Control ²	150	99	99	539.4	24.0	(21.5-25.5)	0.9
	0.001	128	100	99	539.6	24.2	(23.0-25.0)	0.5
	0.01	142	99	98	539.4	24.0	(21.0-25.5)	0.9
	0.1	109	97	97	539.7	24.2	(23.5-25.5)	0.5
	1.0	122	100	100	539.8	24.1	(23.0-25.0)	0.6
	10.0	117	49	49	539.8	24.4	(23.5-25.5)	0.5
2	Control ¹	141	100	99	531.4	24.4	(23.0-25.5)	0.6
	Control ²	146	97	97	535.5	24.2	(23.0-25.5)	0.7
	0.001	148	97	95	531.4	24.1	(21.0-25.5)	0.9
	0.01	130	94	94	537.0	24.5	(23.5-25.5)	0.5
	0.1	150	98	97	534.3	24.1	(23.0-25.0)	0.6
	1.0	123	94	93	533.4	24.2	(22.0-25.0)	0.7
	10.0	135	10	09	536.7	24.2	(23.5-25.0)	0.4
1 & 2 com- bined	Control ¹	240	98	98	534.8	24.4	(23.0-25.5)	0.5
	Control ²	296	98	98	537.4	24.1	(21.5-25.5)	0.8
	0.001	276	99	97	535.3	24.2	(21.5-25.5)	0.7
	0.01	272	96	96	538.3	24.2	(21.0-25.5)	0.7
	0.1	259	98	97	536.6	24.2	(23.0-25.5)	0.6
	1.0	245	97	96	536.5	24.1	(22.0-25.5)	0.6
	10.0	252	28	27	539.3	24.3	(23.5-25.5)	0.4

¹Control test at beginning of series

²Control test at end of series

TABLE 2. Comparison of fertilization success for control eggs and those exposed to 1% methanol, χ^2 values are for 2 x 2 contingency test.

Series	% Fertilized		χ^2	P
	control	1% methanol		
32 sec	98	97	0.30	0.9
30 min	95	96	0.18	0.9

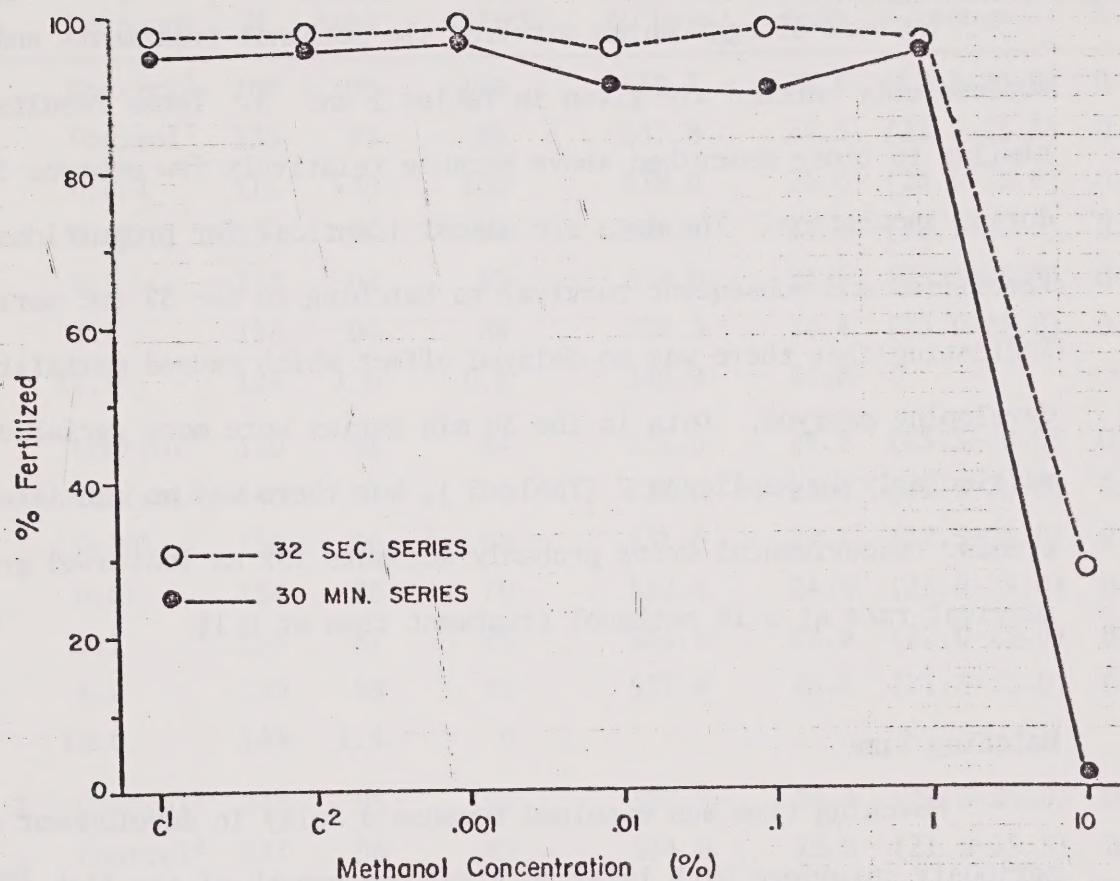


FIGURE 2. Fertilization rates among ova exposed to various methanol concentrations and exposure periods. Control tests at beginning (C¹) and end (C²) of each series are indicated.

may have been present but not counted. However, for the purpose of this study it is irrelevant whether the gametes of the newly-formed zygotes were killed by the 10% concentration.

Survival to Hatching

Numbers of eggs which survived the methanol treatments and successfully hatched are given in Tables 1 and 3. These results are similar to those described above because relatively few embryos died during incubation. The data are almost identical for proportions fertilized and subsequent survival to hatching in the 32 sec series, indicating that there was no delayed effect which caused mortality among developing embryos. Data in the 30 min series were more variable, particularly in Replicate 2 (Table 3), but there was no consistent trends. Experimental error probably accounts for an indicated greater survival rate at a 1% methanol treatment than at 0.1%.

Hatching Time

Hatching time was examined because a delay in development might seriously interfere with later survival and growth of the fish.

Each of the replicates in this experiment was derived from a different set of parents and placed in a separate incubator tray. Several minor differences between these trays were noted, including the time to hatching. The alevins in Replicate 1 hatched significantly later than those in Replicate 2. This difference between means was small and amounted to 5.3 C°-Days or only about one-half day of incubation (Table 4).

TABLE 3. Results of methanol treatments for the 30 min exposure series. Length measurements are based on a sample size of 30 (except in one case, Replicate 1 - 10%, where n=1).

Repli- cate	% Methanol	ALEVINS						
		OVA		% Survival to hatch	Mean hatching (C°Days)	Length (mm)		S.D.
		N	% Fertil- ized			mean	range	
1	Control ¹	104	100	100	539.1	24.4	(22.5-26.0)	0.7
	Control ²	135	99	96	537.8	24.3	(23.5-25.5)	0.6
	0.001	115	100	100	539.6	24.0	(23.0-25.0)	0.6
	0.01	130	98	94	539.7	24.0	(22.5-25.0)	0.5
	0.1	112	94	83	539.0	24.5	(23.5-25.5)	0.5
	1.0	126	94	88	538.3	24.4	(23.0-25.0)	0.5
	10.0	124	1.6	0.8	540.0	24.0	-	-
2	Control ¹	139	91	84	535.6	24.3	(23.5-25.0)	0.5
	Control ²	146	93	90	532.0	23.4	(21.5-25.0)	0.7
	0.001	158	96	88	531.6	24.1	(22.5-25.0)	0.6
	0.01	154	86	79	532.4	24.0	(23.0-25.0)	0.6
	0.1	153	87	65	534.6	23.8	(22.0-25.0)	0.8
	1.0	139	98	95	537.4	24.4	(21.5-25.0)	0.7
	10.0	143	1.4	0	-	-	-	-
1 & 2 com- bined	Control ¹	243	95	91	537.2	24.4	(22.5-26.0)	0.6
	Control ²	281	96	93	534.9	23.8	(21.5-25.5)	0.6
	0.001	273	97	93	535.2	24.0	(22.5-25.0)	0.6
	0.01	284	91	86	536.1	24.0	(22.5-25.0)	0.6
	0.1	265	90	72	536.7	24.2	(22.0-25.5)	0.6
	1.0	265	96	92	537.8	24.4	(21.5-25.0)	0.6
	10.0	267	1.5	0.4	540.0	24.0	-	-

¹Control test at beginning of series

²Control test at end of series

TABLE 4. Comparison of mean hatching times (C°-Days) for replicate samples.

Replicate	C- Degree Days		t	p
	Mean hatching time for replicate	Range of treatment means		
1	539.4	537.8-540.0	8.5	<0.005
1	534.1	531.4-537.4		

Because of this difference, replicates (trays) are treated separately in this section.

The methanol treatments caused no detectable change in hatching times. In Replicate 1 hatching time was extremely precise and uniform both within and between treatments (Figure 3A). The mean time to hatch for all controls and treatments was 539.4° days and the range of values per treatment was small. Over 92% of the eggs in each treatment hatched at $540 \pm 4.6^\circ$ days in the 32 sec series. There was slightly more variability in the 30 min series of Replicate 1, but there too most eggs (78 - 100%) hatched at $540 \pm 4.6^\circ$ days.

In Replicate 2 the eggs hatched over a slightly longer period than in Replicate 1 (Figure 3B). Other minor differences in Replicate 2 include earlier average hatching, more variation between treatments and modal values per cell which varied inconsistently between the 530 and 540° day observations. Otherwise, the overall results between the replicates are similar.

The results obtained in Replicates 1 and 2 were examined for statistical differences by t-test and analysis of variance. First, if the replicates are analyzed separately, there is no significant difference due to the exposure times tested, that is, the mean hatching times were similar in both the 32 sec and 30 min exposures (Replicate 1: $t=1.86$, $p=0.09$; Replicate 2: $t=0.24$, $p=0.8$). Second, if the replicates are compared to each other, there was more variation between replicates

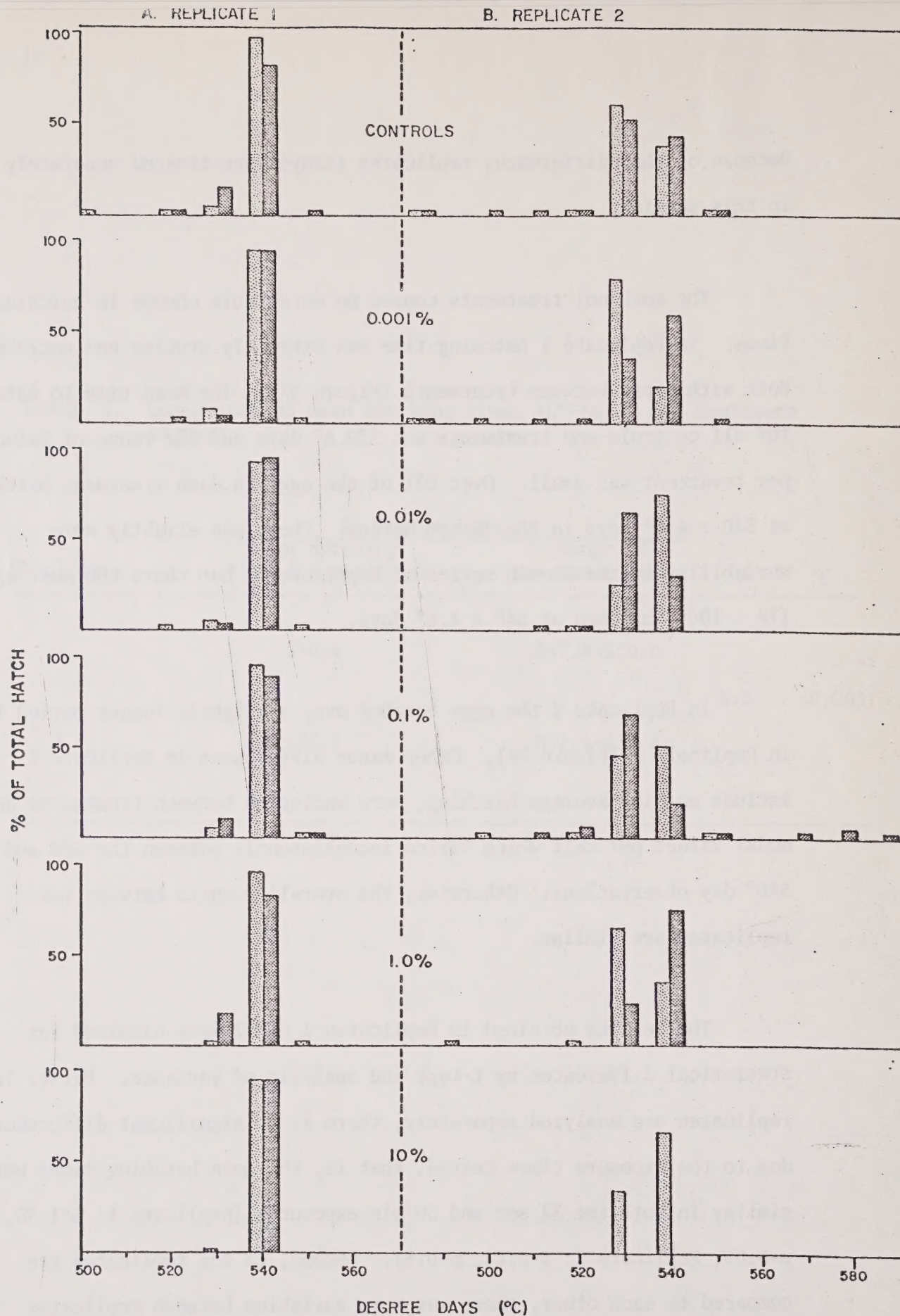


FIGURE 3. Comparison of hatching times for chum salmon in Replicates 1 and 2. Within each replicate, hatching times for fish in the 32 sec exposure series (open bars) and 30 min exposure series (dark bars) are indicated.

than there was between treatments. There was no significant difference in mean hatching time which could be ascribed to methanol concentration in either the 32 sec series ($F=0.16$, $p>0.1$) or 30 min series ($F=0.14$, $p>0.1$). It is clear from these results, especially as illustrated in Figure 3A, that there was no effect on the hatching time of chum salmon eggs due to either 1) the concentrations of methanol or 2) the exposure times tested during fertilization.

Alevin Size at Hatching

Regardless of the methanol treatment, the average length of newly-hatched alevins showed little variation (Tables 1 and 3). The range of mean lengths was only 24.0 - 24.5 mm in the 32 sec series and 23.4 - 24.5 mm in the 30 min series.

Statistical analyses of these data confirm that there was no detectable effect on alevin size associated with methanol concentration. T-test comparisons of mean length at hatching showed that treatments did not differ from control tests. Because of the similarity of results, control tests in this comparison were tested only against "worst case" treatments (*i.e.* the 10% concentration in the 32 sec series and the 1% concentration in the 30 min series due to insufficient numbers surviving in the higher concentration), (Table 5).

Deformities

All newly-hatched alevins were examined for gross physical deformities. In all cases, the incidence of deformities was low, ranging

TABLE 5. Comparison of mean lengths of newly-hatched alevins subjected to various treatments.

Exposure Time	Replicate	Mean Alevin Length (mm)			t	p
		Control	1% conc.	10% conc.		
32 sec	1	24.3		24.4	1.1	0.25
	2	24.4		24.2	0.8	0.4
30 min	1	24.4	24.4		0.2	0.8
	2	24.3	24.4		0.3	0.8

from 0 - 3.1% (Table 6). The most common type of deformity observed as a malformation of the spine, but there was no correlation between incidence of deformity and treatments. An inspection of Table 6 also shows that the incidence of deformity was not related to the two exposure times tested. The overall proportions of deformed alevins in each series were almost identical (1.1 - 1.2%).

Comparison of Control Observations with Other Hatchery Data

Although minor variations in replicate controls were recorded, the control data are considered to be representative of hatchery results for this species. As shown in Table 7, the data are very similar to those reported by other workers for *Oncorhynchus keta*.

TABLE 6. Incidence of deformities among chum alevins subjected to various methanol treatments during the fertilization process. Deformities are listed by number: 1, short lower jaw; 2, two bodies attached to same yolk sac; 3, crooked spine; 4, abnormal yolk sac (dropsy).

Exposure Time	Methanol Concentration	Deformity				Total	No. Examined	%
		1	2	3	4			
32 sec series	Controls	-	-	5	2	7	513	1.4
	.001	1	-	3	-	4	267	1.5
	.01	-	-	1	-	1	264	0.4
	.1	-	-	5	-	5	251	2.0
	1	-	-	-	-	0	249	0.0
	10	-	-	-	-	0	69	0.0
	TOTALS					17	1,615	1.1
30 min series	Controls	-	-	6	-	6	482	1.2
	.001	-	1	5	2	8	254	3.1
	.01	-	-	1	-	1	246	0.4
	.1	-	-	1	-	1	192	0.5
	1	-	-	1	-	1	243	0.4
	10	-	-	-	-	0	1	0.0
	TOTALS					17	1,420	1.2

TABLE 7. Comparison of data obtained during this study with those described by Withler and Morley (1970).

	Methanol expt.- combined controls	Withler and Morley (1970)
1. Incubation temp. (°C)	9.3	11.0
2. Hatching time (° Day) - mean and range	536.3 (531.4-539.8)	541.1 (528.7-557.5)
3. Size at hatching (mm) - mean and range	24.2 (23.4-24.4)	25.2 (24.0-25.6)
4. Physical deformities at hatching (%)	1.1	1.2

DISCUSSION

The results of this experiment should help to alleviate some of the concern that has arisen over possible methanol contamination of streams supporting spawning salmonids. The data demonstrate that, under the experimental conditions tested, brief exposure to methanol up to and including concentrations of 1% does not adversely affect the fertilization process of chum salmon. Continuous exposure to 1% methanol over the entire incubation period, however, will result in 100% mortality for grayling eggs (McMahon and Cartier, 1974).

A 10% concentration of methanol was lethal in most cases, even for short exposures. An average mortality of 73% occurred after an exposure time of only 32 sec; 99.6% died after an exposure time of 30 min. The cause of death in the 10% treatments is not known, but it was observed that this concentration was potent enough to kill some eggs before the end of the 30 min exposure period (these eggs were already turning opaque). McMahon and Cartier (1974) report that even juvenile Arctic char and grayling suffer 100% mortality at this concentration (24 hr test).

Several parameters were examined in this study to detect sub-lethal as well as lethal effects with the lower concentrations of methanol (0.001 - 1.0%): fertilization success, survival to hatching, hatching time, alevin size at hatching, and physical deformities among alevins.

While no significant differences were found between treatment and control observations, there is always a possibility that other sub-lethal effects were not observed. For example, Siefert and Spoor (1974) report that the time when coho salmon, white suckers and brook trout first begin to feed was delayed if the eggs were incubated in an oxygen deficient environment. White suckers in particular had a "distinctly different behavioral pattern" at that stage. Presumably this or any other sub-lethal effect might put the fish at a competitive disadvantage (*e.g.* Mason, 1969). If the methanol experiment is repeated with different species, it would be desirable to extend the period of observation through the time of first feeding.

In summary, from the experimental data which are now available, it appears that eggs, sperm and embryos can withstand a brief exposure to methanol concentrations up to and including 1%. Significant mortality may occur with an increase in either exposure time or concentration of methanol.

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APPENDIX TABLE 1. Analysis of methanol batch used in toxicity experiments.
 (Manufacturer: Fisher Scientific Co., Chemical
 Manufacturing Division, Fair Lawn, New Jersey, 07410).

Certificate of Actual Lot Analysis

Catalog No. A-412	Methanol, A.C.S.	Lot No. 734350
Appearance -----	clear and colorless	
Acetone, Aldehydes -----	pass test	
Boiling Point -----	64.6°C	
Boiling Range		
(1 ml to 95 ml) -----	64.6 - 64.8°C	
(95 ml to dryness) -----	64.8 - 64.9°C	
Water (H ₂ O) -----	0.05%	
Residue after evaporation -----	0.0002%	
Alkalinity (as NH ₃) -----	0.0001%	
Acidity (as HCOOH) -----	0.001%	
Substances Reducing KMnO ₄ -----	P.T.	
Solubility in Water -----	P.T.	
Substances Darkened by H ₂ SO ₄ -----	P.T.	
Color (APHA) -----	5	
FLASH POINT -----	54°F	

